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Profiling *Mycobacterium xenopi* with restriction fragment length polymorphism of insertion element IS*1395*

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SUMMARY

Objectives: The aim of the present study was to assess the usefulness of insertion element IS*1395* for differentiation of *Mycobacterium xenopi*, an increasingly common opportunistic human pathogen.
Methods: Fifty-two isolates obtained from 51 patients in Poland in 1996, 1997, and 1999, were analyzed by IS*1395* restriction fragment length polymorphism (RFLP), and their susceptibilities to 11 anti-tuberculosis drugs were also determined.
Results: IS*1395*-associated banding patterns of the isolates were not highly polymorphic; the RFLP patterns displayed several bands in common. Nevertheless, 44 of the 52 isolates were clearly distinguishable from each other. Only eight strains (15.4%) occurred in four clusters of two identical clones, one of which comprised two isolates obtained from one patient with a 12-month interval. The remaining six patients with clustered strains showed no apparent epidemiologic links with the other patients from the same cluster, and they were most likely infected by the same environmental source. No noticeable difference in RFLP pattern or IS*1395* copy number between drug-sensitive and drug-resistant strains was shown. A high proportion (84.6%) of strains resistant to at least one drug was found, and 7.7% were resistant to more than three drugs.
Conclusions: The stability and utility of IS*1395* for further detailed epidemiological investigations of *M. xenopi* was confirmed and extended.

1. Introduction

Mycobacterium xenopi is a slow-growing non-tuberculous mycobacterium (NTM). It is a well-known opportunistic pathogen commonly associated with human disease, notably with pulmonary infection, causing a clinical presentation said to be similar to disease caused by *Mycobacterium tuberculosis* complex (MTC) species or *Mycobacterium avium-intarcellulare*.[1] Extrapulmonary and disseminated infections have also been recorded.[2,3] A predisposing factor is impaired immunity, either local (e.g., pre-existing pulmonary disease) or systemic (e.g., hematologic malignancy, immunosuppressive medication, or HIV/AIDS).[2,4] Isolation of *M. xenopi* from clinical samples may be indicative of clinical disease, although colonization without disease often occurs. This also applies to HIV-infected patients who can have concurrent illnesses.[5,6]

There are regional differences in the isolation of *M. xenopi*. While uncommon in the USA, it is among the most frequently isolated NTM species in various regions of Canada and Europe.[3,7,8] According to a recent survey of NTM isolates, the frequency of identification of *M. xenopi* has increased worldwide over a 20-year period.[9] Like other potentially pathogenic mycobacteria, *M. xenopi* is widely present in the environment, especially in tap water.[10,11] Dailloux et al. hypothesized that biofilms may be considered reservoirs for the proliferation of mycobacteria in water distribution systems and contributors to the continuous bacteriological contamination of the water via an erosion process.[12] Transmission to humans is believed to originate from the environment, through aerosol inhalation or ingestion. Human-to-human transmission and transmission from animal reservoirs have not been demonstrated. Detailed investigation of animal and environmental reservoirs, as well as the determination of transmission routes and infection modes, requires precise strain-typing techniques.

Restriction fragment length polymorphism (RFLP) upon hybridization to specific probes is a widely used molecular epidemiological method for characterization of strains by genomic fingerprinting. A number of repeated DNA sequences have been successfully used as genetic markers for the identification of mycobacterial species or strain differentiation.[13] An element related to MTC-specific IS*1081* was shown to be present in *M. xenopi*.[14,15] Screening of a partial *M. xenopi* genomic library with an IS*1081* probe resulted in isolation of a new insertion sequence,

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IS*1395*. Despite its potential ability to transpose, IS*1395* was shown to be a useful epidemiological tool for typing of *M. xenopi* strains providing a diversity of RFLP patterns.[16]

In this study, *M. xenopi* clinical isolates from Poland were analyzed by the RFLP method using a probe for IS*1395*. Drug resistance profiles using a wide group of antibiotics were also determined and compared to molecular typing data.

2. Materials and methods

2.1. Patient characteristics

Patient information including date of isolation, HIV status, and standard demographic data were recorded. Fifty-two isolates from 51 patients were analyzed. These patients represent 73% of the patients examined with bacteriologic confirmation of *M. xenopi* infection and 0.7% of all examined patients (positive and negative cultures) at the National Tuberculosis and Lung Diseases Institute (NTLDI) in Warsaw, Poland, in 1996, 1997, and 1999 (Table 1).

Patients originated from different regions of the country. The patients were aged between 27 and 77 years, and 71% were aged between 20 and 60 years. Most of the patients (59%) were men. All were HIV-negative.

2.2. Bacterial strains

The 52 *M. xenopi* strains analyzed in the present study were provided by the NTLDI in Warsaw, which is the national tuberculosis reference laboratory. In one case, a second isolate was obtained with a 12-month interval from a single patient, and these two repeat isolates were included in the analysis. The species identification of the strains was based on standard microbiological tests. All the strains were examined for their susceptibility to 11 drugs: isoniazid, rifampin, streptomycin, ethambutol, ofloxacin, amikacin, ethionamide, capreomycin, clofazimine, rifabutin, and erythromycin using Löwenstein–Jensen medium and the BACTEC 460-TB system (Becton Dickinson, Sparks, MD, USA).

2.3. PCR amplification

Sequence analysis of the insertion element IS*1395* (GenBank accession No. **U35051**) allowed the selection of primers XN1 (5′-ACGAGGCTTTCCAAGTCGCAGG-3′) and XN2 (5′-TCGTTGGTGAC-CAGCGCCAC-3′) at positions 439–460 and 730–749, respectively. The two primers XN1 and XN2 were used to generate a 311-bp probe for RFLP analysis. Purified chromosomal DNA from *M. xenopi* strain 145 was used as a template for the IS*1395* probe amplification. DNAs from *M. xenopi* 50/98 and the reference strain *M. tuberculosis* H37Rv served as controls for PCR specificity.

Amplification reactions were performed with 50-μl volumes containing 2.5 mM each deoxynucleoside triphosphate, 0.2 μM of each primer, 2 U of Taq DNA polymerase (Promega, Madison, WI, USA), and 100 ng of template DNA in the commercial buffer (50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$) (Promega). In addition, the reaction mixtures included dimethyl sulfoxide (DMSO) at three different concentrations: 0%, 3%, or 6%. The cycling parameters consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C or 63 °C for 1 min, and extension at 72 °C for 1 min, with additional 5 min of annealing and 10 min of elongation steps after the last cycle. The PCR programs were performed on an MJ Research thermocycler (Waltham, MA, USA). Amplification products were analyzed by electrophoresis on a 7% polyacrylamide gel and detected by ethidium bromide staining.

Table 1
Numbers of patients investigated at the National Tuberculosis and Lung Diseases Institute in 1996, 1997, and 1999 and enrolled in this study

Year	All patients investigated	Bacteriologic confirmation of *M. xenopi* infection	RFLP analysis of *M. xenopi* strains
1996	2967	24	22
1997	2158	18	14
1999	2452	28	16
Total	7577	70	52

RFLP, restriction fragment length polymorphism.

2.4. RFLP analysis

The 311-bp probe within IS*1395* was prepared as described above and recovered from polyacrylamide gel by overnight elution at 37 °C with 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M ammonium acetate, 10 mM magnesium acetate, and 0.1% sodium dodecyl sulfate (SDS). DNA was purified by phenol/chloroform extraction followed by ethanol precipitation, and labeled with horseradish peroxidase using the ECL system (Amersham).

Chromosomal DNA from the *M. xenopi* isolates was prepared as described previously,[17] and digested with restriction endonuclease *Pvu*II. DNA fragments were resolved by electrophoresis on a 1% agarose gel at 12–15 V/cm and transferred to a nylon membrane (Hybond-N^+; Amersham) using the capillary method. The membrane was hybridized overnight at 42 °C with the probe, then washed twice for 20 min at 42 °C with 6 M urea, 0.4% SDS, and 0.5 × standard saline citrate (SSC), and finally washed twice for 5 min at room temperature with 2 × SSC. The peroxidase-labeled probe was detected with the ECL detection system (Amersham).

The IS*1395* RFLP patterns were examined visually, scanned and compared using ScanPack software (version 3.0; Biometra, Germany). Comparisons of patterns were done by the unweighted pair group method analysis (UPGMA) using the Dice coefficient with the parameter settings at 1.0% band position tolerance with optimization. A molecular cluster was defined as a series of isolates exhibiting 100% identical IS*1395* banding patterns.

3. Results

3.1. Designation and PCR amplification of IS1395 probe

To study the RFLP of the repetitive element among *M. xenopi* strains, primers XN1 and XN2 were designed to produce a 311-bp IS*1395* internal probe (positions 439–749).

Denaturation (94 °C) and extension (72 °C) temperatures, as well as the number of 35 amplification cycles, were maintained throughout two different PCR programs used to obtain the probe. In the preliminary experiments, amplification reactions were performed at the annealing temperature of 60 °C. Under these reaction conditions, a variable number of non-specific products were seen in DNA extracted from two *M. xenopi* strains in addition to the expected one. Moreover, non-specific amplicons (including that of 311 bp) appeared in the control sample containing DNA from *M. tuberculosis* H37Rv (data not shown). Increasing the stringency to 63 °C allowed for the amplification of the expected 311-bp fragment within IS*1395* in DNA templates from *M. xenopi* only (Figure 1, lanes 5–6 and 8–9), and yielded very few non-specific products of both wrong sizes and very low concentrations with DNA template from *M. tuberculosis* H37Rv (Figure 1, lane 3). Also, it proved necessary to include DMSO in a reaction mixture. This agent facilitates denaturation of double-stranded DNA (dsDNA), especially with high G+C content characteristic of mycobacteria. In the absence of DMSO, the expected 311-bp fragment of IS*1395* was not produced (Figure 1, lanes 4 and 7).

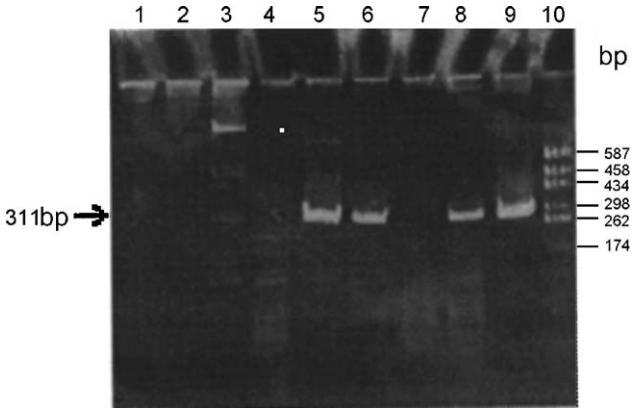

Figure 1. Polyacrylamide gel electrophoresis of PCR products from *Mycobacterium tuberculosis* H37Rv (lanes 1–3), *Mycobacterium xenopi* 145 (lanes 4–6), and *M. xenopi* 50/98 (lanes 7–9) amplified at 63 °C annealing with primers XN1 and XN2 and using no DMSO (lanes 1, 4 and 7), 3% DMSO (lanes 2, 5 and 8), or 6% DMSO (lanes 3, 6 and 9). Lane 10, molecular size standard (pUC19 digested with *Hae*III). Arrow indicates the position of a 311-bp IS*1395* fragment.

3.2. IS1395 RFLP analysis of M. xenopi strains

The 52 strains were analyzed by RFLP with IS*1395* as a hybridization probe. *Pvu*II was used for digestion of genomic DNA as IS*1395* did not contain a corresponding restriction site. The copy number of IS*1395* in each of the strains was determined from the number of bands hybridizing to the probe, and ranged from 6 to 13 (Figure 2). The majority of the strains (n = 46; 88.5%) contained 9–12 copies, with a mean of 10 bands.

The DNA fingerprints generated were not highly variable; the RFLP patterns displayed several bands in common. In the region of 2.5 kbp, all the studied strains showed three bands. Also, a computer-assisted similarity analysis demonstrated seven clusters of patterns with a high (~95%) level of relatedness (Figure 2). Five of these comprised two patterns each that differed by the presence of one additional band. Despite the relatively low diversity of the patterns, the IS*1395* RFLP technique allowed for sufficient discrimination of the strains tested. Forty-eight different IS*1395* DNA fingerprint patterns were observed in the 52 strains analyzed. Of the 48 patterns, only four were identical for two strains each, whereas the remaining 44 patterns (92% of strains) were found only once each in this investigation (Figure 2). Three pairs of identical strains were isolated at different times from different patients presumably representing cases of infection from a common, most likely environmental, source. The fourth cluster comprised two isolates obtained with a 12-month interval from one patient, but it did not lead to changes in the RFLP pattern (Figure 2). This observation confirmed sufficient stability of IS*1395* as an epidemiological marker useful in typing of *M. xenopi* strains.

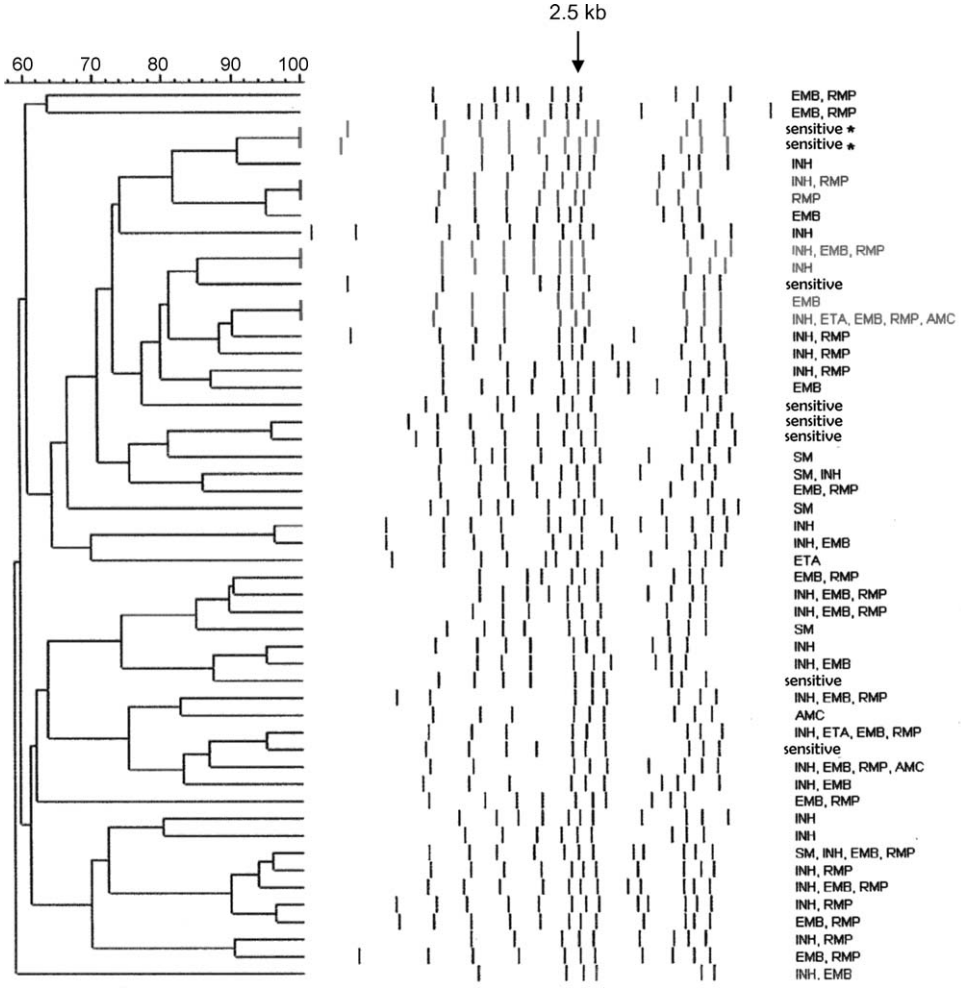

Figure 2. IS*1395* RFLP patterns of the 52 *Mycobacterium xenopi* isolates and the corresponding dendrogram. Drug resistance profiles of the analyzed isolates are given on the right. The similarity among the patterns is indicated as a percentage above the dendrogram. *Two consecutive isolates from the same patient.

Table 2
Drug resistance profiles of *Mycobacterium xenopi* strains

Group[a]	Drug	No. (%) of resistant strains isolated			
		1996	1997	1999	Total
I	RMP	0	0	1 (6.3)	1 (1.9)
	EMB	0	0	3 (18.8)	3 (5.8)
	AMC	0	1 (7.1)	0	1 (1.9)
	ETA	1 (4.5)	0	0	1 (1.9)
	SM	3 (13.6)	0	0	3 (5.8)
	INH	5 (22.7)	2 (14.3)	0	7 (13.5)
II	INH, RMP	2 (9.1)	2 (14.3)	1 (6.3)	5 (9.6)
	INH, SM	1 (4.5)	0	0	1 (1.9)
	INH, EMB	1 (4.5)	3 (21.4)	1 (6.3)	5 (9.6)
	RMP, EMB	1 (4.5)	0	6 (37.5)	7 (13.5)
III	INH, RMP, EMB	1 (4.5)	1 (7.1)	3 (18.8)	5 (9.6)
	INH, RMP, AMC	0	1 (7.1)	0	1 (1.9)
IV	Resistant to more than three drugs	0	3 (21.4)	1 (6.3)	4 (7.7)
Total		15/22 (68.2)	13/14 (92.9)	16/16 (100)	44/52 (84.6)

RMP, rifampin; EMB, ethambutol; AMC, amikacin; ETA, ethionamide; SM, streptomycin; INH, isoniazid.
[a] Groups I, II, III, and IV include strains resistant to one, two, three, or more than three drugs, respectively.

3.3. Correlation between IS*1395* RFLP patterns and drug resistance

Among 52 *M. xenopi* strains, only eight (15.4%) were susceptible to all 11 drugs tested. The drug resistance patterns of the remaining 44 (84.6%) strains are given in Table 2. Sixteen (30.8%) strains were found to be resistant to at least one anti-tuberculosis drug, and 28 strains (53.8%) were resistant to two or more drugs. There was a large increase in the number of resistant strains identified in 1999 (100%) compared to 1996 (68.2%), and most of these (37.5%) were resistant to both rifampin (RMP) and ethambutol (EMB).

IS*1395* RFLP patterns of 44 drug-resistant strains were compared with those of eight drug-sensitive strains. The results showed no noticeable difference in banding pattern or copy number of IS*1395* between drug-sensitive and drug-resistant strains. Strains with different drug resistance profiles were found in the same clusters. One cluster consisted of two sensitive isolates consecutively obtained from one patient. On the other hand, strains with identical drug resistance profiles displayed different RFLP patterns (Figure 2). These results suggest that resistance of *M. xenopi* to one or more anti-tuberculosis drugs does not correlate with the IS*1395* DNA fingerprints.

4. Discussion

Among the NTM, *M. xenopi* is an increasingly common cause of both pulmonary and disseminated disease in humans, yet very little is known about its molecular epidemiology, the possibility of human-to-human transmission of these mycobacteria, and implications for disease control. Since there is no obligation to report NTM infections in Poland, a systematic review of NTM is lacking. The available data covering the studied period revealed that *Mycobacterium kansasii*, *M. avium* complex, and *M. xenopi* were the most frequent NTM isolates in Poland in 1991–2001.[18,19] In the present study, 52 clinical isolates of *M. xenopi* were subjected to molecular characterization based on the RFLP of the insertion element IS*1395*.

IS*1395* is a member of the *Staphylococcus aureus* IS*256* family of insertion sequences. It displays 86% nucleotide sequence and 89% amino acid identity with MTC-specific IS*1081*.[16] The high percentage homology between these IS*256* family members was the most probable cause of unspecific PCR amplification of a 311-bp DNA fragment in *M. tuberculosis* at the annealing temperature of 60 °C in the present study. To eliminate this false-positive result during amplification of IS*1395* probe for RFLP analysis, we conducted the reaction at higher stringency (63 °C), which yielded the expected product in DNA templates from *M. xenopi* only. Previous investigation of the host range of IS*1395* by Southern blot analysis revealed additional IS*1395*-related IS*256* repeated sequences in *M. avium*, *Mycobacterium gordonae*, and *Mycobacterium celatum*.[16] Similarly, homology between IS*3*-like elements from several NTM species and a central region of MTC-specific IS*6110*, which overlaps the internationally agreed RFLP probe, has been reported.[20] This homology was probably the cause of false-positive results we observed previously in an IS*6110*-based PCR assay for MTC when performed at 60 °C or 55 °C annealing temperatures.[17] Results of our previous study and this study on PCRs performed at high stringencies (65 °C and 63 °C, respectively) support the observations of others – that insertion sequence-targeted PCR assays should be carefully designed and evaluated to specifically detect mycobacterial species.[20,21]

Data on DNA polymorphism within *M. xenopi* are scarce in the literature and include restriction fragment analysis and hybridization with probes of the insertion elements IS*1395*[16] or IS*1081*[15] to detect RFLPs among the strains. RFLP patterns were found to be identical using IS*1081* or IS*1395* as probes.[16] This study further evaluated the IS*1395* as an epidemiological marker on a large set of *M. xenopi* strains and extended previous data. In accordance with observations from an RFLP study of *M. xenopi* isolates from the USA,[15] the isolates investigated in the present study carried from 6 to 13 copies of the IS*1395* element (median 10). This is less than the 3–18 copies (median 14) described in a French study.[16] The authors also reported that 15 out of 19 studied strains showed either five or two copies of IS*1395* in the region of 2.5 kbp. Interestingly, all 52 isolates tested here had three bands in that region, indicating their close relationship. Nevertheless, DNA fingerprinting analysis revealed 48 distinct patterns among the strains studied, which is similar to the level of polymorphism described by others. Collins observed seven different IS*1081* RFLP patterns among 10 *M. xenopi* strains isolated from humans.[15] Picardeau et al., using the IS*1395* probe, detected specific RFLP patterns for 19 clinical strains of *M. xenopi* despite the generally low level of polymorphism generated.[16]

In the present study, four pairs of identical IS*1395* RFLP patterns were encountered. One of these clusters comprised two consecutive isolates obtained with a 12-month interval from one patient, thus confirming the stability of IS*1395* and its utility as an epidemiological marker. It is generally accepted that *M. tuberculosis* strains with unique RFLP profiles upon hybridization with the IS*6110* probe indicate cases of endogenous reactivation of previously acquired infection, and clustered strains are likely to represent epidemiologically linked cases of recently transmitted

disease.[22] In contrast, whereas clusters of cases tend to occur, active transmission of NTM has never been demonstrated and environmental sources of infection are likely, yet it cannot be excluded.[1] Tracing the transmission routes of NTM is very difficult due to their ubiquitous presence in the environment. Based on the available epidemiological data, no direct link between clustered patients in this study could be proved and they were most likely infected by the same environmental source of *M. xenopi*. Since data on the clinical significance of the infections were not available, it cannot be excluded that some of the cases might have resulted from contamination of clinical specimens and nosocomial acquisition from the hospital water supply.[23]

NTM species have been shown to have natural resistance to many anti-tuberculosis drugs that, together with the frequently observed lack of correlation between in vitro drug susceptibility testing and clinical response, makes treatment difficult.[1,24] In the present study, resistance to at least one anti-tuberculosis drug was found in 84.6% of the 52 *M. xenopi* strains, but in 100% of strains from 1999. Monoresistance to isoniazid (INH) as well as resistance to both RMP and EMB were predominant (13.5% each). The strains resistant to three drugs accounted for 11.5% (resistance to INH, RMP, and EMB was predominant in this group and accounted for 9.6%), whereas 7.7% of all the tested strains were resistant to more than three anti-tuberculosis drugs. The four strains comprising this latter group had different resistance patterns, but all were resistant to INH, RMP, and EMB. Similar resistance profiles were encountered with comparable frequencies in other studies.[6,24] Recent data suggest that INH is not as effective as other drugs against pulmonary *M. xenopi*,[25] but RMP and EMB, which are recommended as the cornerstone of treatment for this pathogen, did not prove more effective in susceptibility testing in this study. Nevertheless, in vitro resistance does not preclude an in vivo efficacy, hence RMP and EMB cannot be excluded from the first-line treatment of *M. xenopi* infections. Also, we did not observe gross differences in RFLP patterns between drug-resistant and drug-sensitive strains, a finding similar to our previous results on *M. tuberculosis*.[26]

This study demonstrates on a large set of *M. xenopi* strains that RFLP analysis with the IS*1395* probe provides a useful tool for strain typing, based on variations of both copy number and distribution of this insertion element within the genome. The results indicate a lack of correlation between RFLP type and drug resistance profile for *M. xenopi* strains. Due to the paucity of data available for this increasingly common pathogen, further studies that focus on better delineating the transmission routes and identifying sources of infection are needed.

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Conflict of interest: No conflict of interest to declare.

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